VOLUME 30 APRIL 1952 NUMBER 4

ENGIN. LIB.
T
1
.C22

Canadian Journal of Technology

Editor: G. A. LEDINGHAM

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

UNIVERSITY OF MICHIGAN LIBRARIES

Engineering
T
1
.C221

CANADIAN JOURNAL OF TECHNOLOGY

(Formerly Section F, Canadian Journal of Research)

The CANADIAN JOURNAL OF TECHNOLOGY is published monthly by the National Research Council of Canada under the authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research. Matters of general policy are the responsibility of a joint Editorial Board consisting of members of the National Research Council of Canada and the Royal Society of Canada.

The National Research Council of Canada publishes also: *Canadian Journal of Botany, Canadian Journal of Chemistry, Canadian Journal of Medical Sciences, Canadian Journal of Physics, Canadian Journal of Zoology.*

The CANADIAN JOURNAL OF TECHNOLOGY and the CANADIAN JOURNAL OF CHEMISTRY have been chosen by the Chemical Institute of Canada as its medium of publication for scientific papers.

EDITORIAL BOARD

Representing
NATIONAL RESEARCH COUNCIL

DR. J. H. L. JOHNSTONE (*Chairman*),
Professor of Physics,
Dalhousie University,
Halifax, N.S.

DR. OTTO MAASS,
Macdonald Professor of
Physical Chemistry,
McGill University,
Montreal, P.Q.

DR. CHARLES W. ARGUE,
Dean of Science,
University of New Brunswick,
Fredericton, N.B.

DR. A. G. McCALLA,
Department of Plant Science,
University of Alberta,
Edmonton, Alta.

Representing
ROYAL SOCIETY OF CANADA

DR. G. M. VOLKOFF,
Professor of Physics,
University of British Columbia,
Vancouver, B.C.

DR. J. W. T. SPINKS,
Dean, College of Graduate
Studies,
University of Saskatchewan,
Saskatoon, Sask.

Section III

DR. H. S. JACKSON,*
Head, Department of Botany,
University of Toronto,
Toronto, Ont.

DR. E. HORNE CRAIGIE,
Department of Zoology,
University of Toronto,
Toronto, Ont.

Section V

Ex officio

DR. LÉO MARION (*Editor-in-Chief*),
Division of Chemistry,
National Research Laboratories,
Ottawa.

DR. H. H. SAUNDERSON,
Director, Division of Information Services,
National Research Council,
Ottawa.

Representing
THE CHEMICAL INSTITUTE OF CANADA

DR. H. G. THODE,
Department of Chemistry,
McMaster University,
Hamilton, Ont.

Manuscripts should be addressed to:

DR. LÉO MARION,
Editor-in-Chief,
Canadian Journal of Technology,
National Research Council,
Ottawa, Canada.

Each manuscript should be typewritten, double-spaced, and the original and one extra copy submitted (see **Notice to Contributors** inside of back cover).

Subscriptions, renewals, and orders for back numbers should be addressed to:

Administrative Services,
National Research Council,
Ottawa, Canada.

Subscription rate: $4.00 a year; single numbers: 50 cents. Special rates can be obtained for subscriptions to more than one of the Journals published by the National Research Council.

**Deceased.*

Canadian Journal of Technology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOL. 30 APRIL, 1952 NUMBER 4

A NOVEL HIGH VACUUM FURNACE

BY E. J. CAULE

ABSTRACT

A furnace is described which enables small specimens to be heated in a vacuum. It is fast in action and has definite advantages of ease in use, simplicity in construction and adaptability to many types of work.

In research on the high temperature oxidation of iron the need was found for a device capable of heating small metal specimens to high temperatures in a "hard" vacuum. A high-frequency induction furnace would perform the operation, but was unnecessarily bulky, over-powered, and costly. In contrast the simple apparatus developed is compact, uses but a few watts, and can be built for a materials cost of about 10 dollars. A competent glass-blower can assemble it in half a day.

The mode of action of the furnace is that radiant heat leaving a "Globar" heater at the focus of a parabolic mirror is sent as a roughly parallel beam to another parabolic mirror and is there focused on the metal specimen. The two mirrors form the ends of the vacuum chamber and are connected by a short cylinder, through the walls of which the supports for the heater and the specimen are inserted on standard-taper joints. The supports for the specimen are also the two wires of a chromel–alumel thermocouple, the junction of which is spot-welded to the specimen. The wires are wound around a curved glass support which places their junction at the focus. The usual form of specimen in this work is a square of foil, whose face away from the heater is blackened with carbon.

The model now in use in these laboratories is illustrated in the accompanying figure. The ends were shaped and blown on a lathe to fit a template prepared of asbestos board from a graph of the form $y = 0.16x^2$, the units being centimeters. The shaping was not too tedious, since no attempt was made to secure a point focus; for the same reason, the fit of the template was to the outside of the glass rather than to the inside. After assembly in the unit the mirrors were formed by coating with aluminum by evaporation from a tungsten filament, with mica shielding so arranged as to keep a central band of the cylinder clear for about one inch of length. The "Globar" was turned and filed

[1] *Manuscript received December 14, 1951.*
Contribution from Division of Chemistry (Applied), National Research Council, Ottawa, Canada. Issued as N.R.C. No. 2675.

down from one of those commonly used in infrared spectrometers; in each end there was a concavity, into which fitted a ball on the end of a platinum wire. The platinum wires were spot welded to tungsten leads, whose springiness kept the globar in place.

The electrical circuit is simple, consisting of a 60 cycle variable power supply, an ammeter, a "glow coil" to act as a ballast resistor, and the "Globar", all in series. The current is in the range 0–1.5 amp.

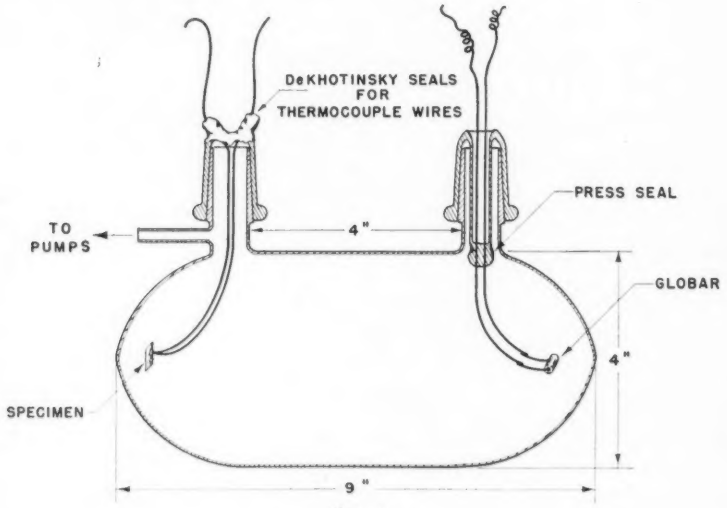

FIG. 1.

In daily use for several months the furnace has proved highly satisfactory; it is rugged, easy to operate, and meets every requirement of the research project in which it is being used. Its present function is to enable specimens of pure iron sheet to be oxidized for definite lengths of time at known temperatures and gas pressures. The metal specimen, which is usually 12 mm. long, 10 mm. wide, and 0.25 mm. thick, is first heated in vacuum to a fixed temperature, usually 400° C. Air or oxygen is then suddenly admitted to a fixed pressure and the temperature of the specimen drops sharply by about 45° and then remains constant; after a predetermined time the current to the "Globar" is shut off and pumping begun. To secure a definite oxidation time it is necessary that the specimen cool quickly when the current is stopped. It has been found that the decay of the temperature of the specimen is exponential, following the equation $\frac{\Delta T}{\Delta T_0} = e^{-kt}$, where ΔT is the temperature increment above room temperature at any time t and ΔT_0 is the temperature increment at time zero, the instant when the current is stopped. With the arrangement illustrated the value of k is 0.0205 reciprocal seconds, or, in other

terms, such that the rate of cooling at 375° C. is about 7 degrees per second, or 0.3 calories per second. In 20 sec. the specimen cools about 100°, thus sharply slowing down the oxidation reaction.

Temperature control is excellent; the "Globar" when hot responds quickly to adjustment of the voltage supply and slow drifts in the line voltage can be corrected manually. In the course of this work it has not been necessary to regulate the temperature more closely than is possible by hand, but temperatures are still constant to ±1°. The highest temperature attained in the course of preliminary experimentation was 670° C., at which an aluminum foil specimen melted.

It is believed that heating is uniform over the area of the specimens. The aluminum specimen mentioned above melted and rounded uniformly. The iron specimens have uniform oxide coatings as shown by the interference colors. Also special experiments in which the samples were moved around on a ball joint or on the standard-taper joint have shown that the focus is not a sharp point but a region comparable in cross section with the specimen.

Remarks on the efficiency of heat transfer from the heater are largely academic, since it has been calculated that the power input is less than 10 watts. The major heat losses are through the uncoated portion of the cylinder and through the support wires; the mirrors remain cool to the touch. There are some convection effects when gas is introduced; any cooling can be compensated for in a few seconds by voltage regulation.

In addition to the rather limited use for the furnace mentioned above, its simplicity and ease of use will probably recommend it for many other applications. The main limitation on adaption is that the supports for other specimens must not be too highly conducting.

ACKNOWLEDGMENT

Thanks are due to Mr. G. Ensell for aid in design and construction of this furnace.

DETERMINATION OF FREE CHLORINE IN WATER BY MEANS OF *o*-DIANISIDINE DIHYDROCHLORIDE REAGENT[1]

By K. L. McEwen[2] and G. A. Grant[3]

ABSTRACT

A colorimetric method for the determination of traces of free chlorine in water, using *o*-dianisidine dihydrochloride has been developed. The test will detect concentrations of chlorine as low as 0.1 p.p.m. Of the ions occurring in water, it was found that nitrite and ammonium ions seriously interfere, but that ferric ion does not. The pH can vary from 4.0 to 6.0 without critically affecting the development of color. The noninterference of iron, and, to some degree, of pH, the stability of the solid reagent as well as the simplicity of the test, make it desirable as a field method.

INTRODUCTION

The present investigation has had as its aim the development of a visual colorimetric method for detecting traces of free chlorine in water. The method must be simple to carry out, and the indicator used must be unaffected by ions commonly found in water and must be a stable solid at temperatures ranging up to 60° C.

Several methods have already been devised for detecting traces of free chlorine in water, and are reviewed by Houghton (3) and Love (4). The *o*-tolidine method, which was developed by Ellms and Hauser (2), has the desired sensitivity and has the advantage of simplicity. However, the test is not specific for chlorine, as ferric ion, nitrite ion, and manganic ion interfere. A method has been suggested whereby a correction would be made for interfering ions. However, this method does not give a very clear-cut differentiation of chlorine at low concentrations. It would therefore be unsuitable for simple, visual determinations. The *p*-aminodimethylaniline test described by Byers (1) for free chlorine which was put on a quantitative basis by Palin (6), is interfered with by manganese and, more seriously, by copper. The latter interference can be inhibited by use of hexametaphosphate. However, a simple visual determination would be difficult with such interfering substances present. Another method for detecting free chlorine has been reported by Taras (7). This method consists of observing the bleaching effect of the free chlorine on methyl orange, methyl yellow, or methyl red. Methyl orange was found to give the best results, and although free of interference from iron, the reaction is unsuitable as it is evident that pH would have a critical effect.

A method for determination of free chlorine was developed by Milton (5), which does not depend upon an oxidation–reduction indicator. The test

[1] *Manuscript received November 21, 1951.*
Contribution from the Defence Research Chemical Laboratories, Ottawa, Canada.
Issued as D.R.C.L. Report No. 79A.
[2] *Graduate Assistant.*
[3] *Research Scientist.*

consists of reacting free chlorine with sodium cyanide, allowing the resulting cyanogen chloride to react with pyridine to form a quaternary compound, which, on subsequent condensation with aromatic amines, produces ring fission and the formation of intensely colored dianyl derivatives. The difficulty with this reaction, which is reported to be extremely sensitive, is the ability of pyridine to form complexes with many ions which might be present in natural water.

In the present investigation advantage was taken of the oxidizability of the benzidine-type compounds. In conjugated molecules, such as benzidine, there are nonlocalized π electrons which lower the oxidation potential. The oxidation potential is further lowered by saturated substituents such as OH, OCH_3, NH_2. It should therefore be possible to choose a benzidine-type compound which would be oxidized by free chlorine, but not, for example, by ferric iron. After a preliminary survey of benzidine type compounds, *o*-dianisidine dihydrochloride was chosen for this investigation.

EXPERIMENTAL

Preparation of Reagents

The *o*-dianisidine dihydrochloride was prepared by treating a saturated solution of E.K. practical *o*-dianisidine in acetone with dry hydrogen chloride, while it was being cooled in an ice bath. The precipitated material was filtered, washed with acetone, and dried. The dry dihydrochloride was ground into a powder and rewashed with acetone. (Yield 89%.) The product must be pure white, as colored contaminants promote its decomposition. The stability of the *o*-dianisidine dihydrochloride was tested, and it was found that a sample which had been kept at 60° C. for a month had not deteriorated appreciably. For the free chlorine analysis a 0.1% aqueous solution of *o*-dianisidine dihydrochloride was used.

A stock chlorine solution was prepared by passing pure chlorine into distilled water. The solution was standardized daily by titration with sodium thiosulphate. The standardized stock solution was then diluted to the required concentrations in volumetric flasks for use in obtaining the standardization curve. In all phases of this investigation c.p. reagents were used.

Methods

In estimating free chlorine, 1 ml. of the 0.1% indicator solution was added to 10 ml. of the chlorine water to be tested. The color was allowed to develop for 25 min., then the solution was transferred to an absorption cell with a 2 cm. light path. The color intensity was measured in a Beckmann Spectrophotometer, employing a radiation of wave length 450 mμ.

RESULTS

In developing an analytical procedure involving an oxidation–reduction indicator, the effects on the reaction produced by a number of factors such as pH, concentration, contaminants, and time must be investigated. The

results are given below for factors investigated which were considered critical in the reaction between free chlorine and *o*-dianisidine dihydrochloride.

An absorption curve was obtained for the reaction products resulting from adding *o*-dianisidine hydrochloride to a chlorine solution. It was found that the maximum absorption was at a wave length of 450 mμ (Fig. 1). The effect

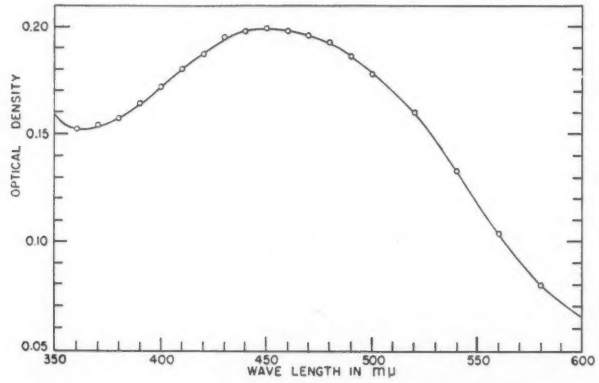

FIG. 1. The absorption curve for chlorine – *o*-dianisidine dihydrochloride reaction products.

of light and of age of the indicator solution on the development of color intensity were studied. As may be seen in Table I, the color was only slightly more intense when the reaction proceeded in daylight, than in the dark; and from Table II it is evident that, as the age of the indicator solution increased, the optical density at 450 mμ of the reaction products with free chlorine

TABLE I

EFFECT OF LIGHT ON THE CHLORINE *o*-DIANISIDINE DIHYDROCHLORIDE REACTION

Chlorine concentration in p.p.m.	Optical density	
	Light	Dark
2.0	0.336	0.334
2.5	0.438	0.436
3.0	0.567	0.566

increased. The effect of concentration of the *o*-dianisidine solution was studied using indicator solutions containing 0.1%, 0.5%, and 1.0% dianisidine hydrochloride. As is shown in Table III, the maximum optical density was obtained when the indicator solution contained 0.1% *o*-dianisidine. It is also evident that as the concentration of the indicator solution was increased from 0.1% to 0.5% or over, a decrease of about 60 mμ in the wave length of the absorption maximum occurred, as is shown in Fig. 2. The rate of development

TABLE II

EFFECT OF THE AGE OF THE *o*-DIANISIDINE DIHYDROCHLORIDE SOLUTION ON OPTICAL DENSITY

(Chlorine concentration 2.3 p.p.m.)

Age of indicator solution, days	Estimated p.p.m. of Cl_2	% Recovery of Cl_2
0	2.30	100
1	2.40	104
5	2.43	106
7	2.45	107
8	2.50	109

TABLE III

EFFECT OF CONCENTRATION OF *o*-DIANISIDINE DIHYDROCHLORIDE SOLUTION ON OPTICAL DENSITY

Concentration of dianisidine solution, %	Optical density		
	Soln. *A*	Soln. *B*	Soln. *C*
0.1	0.285	0.485	0.682
0.5	0.213	0.356	0.444
1.0	0.192	0.309	0.423

NOTE: *Solution A = 1.83 p.p.m. free chlorine.*
Solution B = 2.68 p.p.m. free chlorine.
Solution C = 3.52 p.p.m. free chlorine.

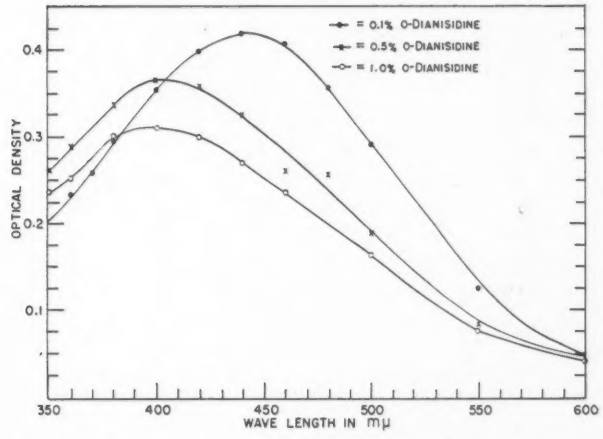

FIG. 2. The effect of concentration of *o*-dianisidine dihydrochloride on the absorption curve.

of color and the stability of the reaction products was studied by measuring the color intensity after different intervals of time. From Table IV it can be seen that the maximum color is produced in about 25 min.

TABLE IV

STABILITY OF REACTION PRODUCTS OF THE FREE CHLORINE TEST
(Concentration of chlorine solution 1.5 p.p.m.)

Time, min.	Optical density
15	0.183
25	0.184
35	0.184
55	0.184

A calibration curve was obtained showing the increase of optical density with increasing concentration of free chlorine. From Fig. 3 it is seen that Beer's Law is obeyed up to a concentration of about 1 p.p.m. of free chlorine; then the slope of the curve increases abruptly and the relation again becomes linear.

The effect of pH and of certain ions was also studied. The effect of pH was studied over a pH range of 3.45 to 5.60. A buffer solution consisting of

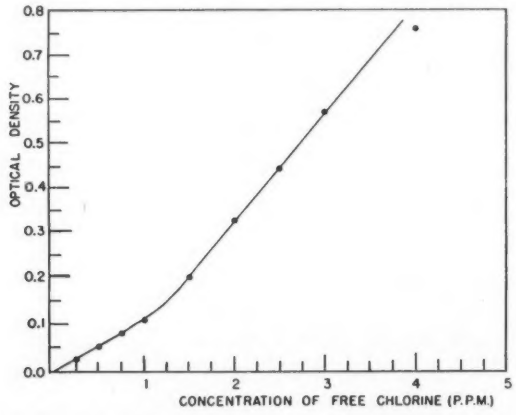

FIG. 3. Relation between optical density of *o*-dianisidine dihydrochloride – chlorine solutions and chlorine concentration.

acetic acid and sodium acetate was used (8). From Table V it is apparent that as the pH increased there was a slight increase in optical density. The results are given in Table VI of the effects of certain ions, which occur naturally in water. It was found that nitrite and ammonium salts seriously affect the test, the nitrite because it reacts to give a colored blank solution, and the ammonium probably by decreasing the oxidation potential of the reaction (5).

The nitrite ion and ammonium are oxidized to the nitrate ion by the hypochlorous ion. Therefore, the low recoveries found when these ions are added to known solution of free chlorine are not due to errors in the determination

TABLE V

EFFECT OF pH ON COLOR INTENSITY
(Concentration of chlorine solution 3 p.p.m.)

pH	Estimated free chlorine p.p.m.	% Recovery of free Cl_2
3.45	2.10	70
4.00	2.36	79
4.95	2.70	90
5.60	2.80	93

TABLE VI

EFFECT OF OTHER IONS ON COLOR INTENSITY
(Concentration of chlorine solution 3 p.p.m.)

Ion	Concentration of ion p.p.m.	pH	Estimated free Cl_2	% Recovery
Fluoride (as Na salt)	5	4.8	3.38 p.p.m.	113
Nitrite (as Na salt)	5	4.2	0.15	5
Calcium (as nitrate)	500	4.3	3.50	117
Magnesium (as nitrate)	200	4.25	3.60	120
Bicarbonate (as Na salt)	500	7.35	3.35	112
Nitrate (as Na salt)	100	4.25	3.10	103
Chloride (as Na salt)	1000	7.00	3.01	100
Iron (ferric) (as chloride)	10	3.6	2.99	100
Ammonium (as carbonate)	100	8.7	0.75	25

of the free chlorine, but are due to the free chlorine being employed to oxidize the nitrite and ammonium ions.

o-Dianisidine dihydrochloride has several advantages as an indicator for free chlorine. The reagent is stable and ferric ion does not seriously interfere with color development. These facts, and the simplicity of the method, make it suitable as a field method for detection and analysis.

The shift in absorption maximum which occurs when the concentration of the *o*-dianisidine dihydrochloride solution is varied makes it imperative to prepare indicator solutions of 0.5% or over when using a spectrophotometer employing monochromatic radiation, for determining free chlorine content. The above-mentioned change in the absorption spectrum is probably due to a shift in the distribution of reaction products as the amount of indicator added is varied.

REFERENCES

1. BYERS, D. H. and MELLON, M. G. Ind. Eng. Chem., Anal. Ed. 11: 202-203. 1939.
2. ELLMS, J. W. and HAUSER, S. J. Ind. Eng. Chem. 5: 915-917. 1913.
3. HOUGHTON, G. U. Analyst, 75: 180-184. 1950.
4. LOVE, S. K. Anal. Chem. 23: 253-257. 1951.
5. MILTON, R. F. Nature, 164: 448. 1949.
6. PALIN, A. T. Analyst, 70: 203-207. 1945.
7. TARAS, M. Anal. Chem. 19: 342-343. 1947.
8. VOGEL, A. L. A text book of quantitative inorganic analysis. Longmans, Green and Company. 1942. p. 808.

EXTRACTION OF POLYHYDROXY COMPOUNDS FROM DILUTE AQUEOUS SOLUTIONS BY CYCLIC ACETAL FORMATION

IV. RECOVERY OF *n*-BUTYRALDEHYDE[1]

By J. M. Roxburgh

ABSTRACT

Chemical and physical losses of *n*-butyraldehyde in the extraction of glycerol by the formation of the cyclic *n*-butyral have been evaluated. Solubility data for the system *n*-butyraldehyde–water under varying conditions have been determined. Solvent extraction has been investigated as an alternative to distillation as a method for the recovery of *n*-butyraldehyde from aqueous solutions. Toluene is shown to be the most satisfactory solvent tested, with a distribution coefficient of 12.83, and the subsequent separation by distillation from the toluene is readily achieved. In a single batch extraction under conditions chosen as "standard" the experimental loss of *n*-butyraldehyde is shown to be about 11% in laboratory scale equipment. This loss is considered to be a maximum value which could be considerably reduced by the use of continuous equipment and reduced pressure in distillation.

INTRODUCTION

The extraction of glycerol from water by the formation of the cyclic acetal with *n*-butyraldehyde has been discussed in prior papers in this series (8, 9, 10). Since losses of *n*-butyraldehyde are important to the economics of such a process, in this paper data are given for physical and chemical losses, and investigations have been made to determine methods by which they may be reduced to a minimum.

EXPERIMENTAL

Analytical Methods

The determination of glycerol and its cyclic *n*-butyral have been described in previous papers (8, 10). Water was determined with Carl Fischer reagent as described by Bryant, Mitchell, and Smith (2) using hydrocyanic acid in dry pyridine to prevent interference from the butyraldehyde.

Butyraldehyde was normally determined by reaction with excess bisulphite. Samples for the determination contained not more than 0.4 millimoles. Volume of the sample was made up to about 20 ml. with water, or with water and ethanol if the sample was not soluble in water alone, but more than 5 ml. of the alcohol was found to interfere with the starch–iodine end point. Five milliliters of 4% aqueous sodium metabisulphite was added and the mixture allowed to stand 30 min. at room temperature in a glass stoppered Erlenmeyer flask, after which the excess bisulphite was neutralized with approximately 1 *N* iodine, the titration being taken exactly to the starch end point with

[1] *Manuscript received November 8, 1951.*
Contribution from the National Research Council, Prairie Regional Laboratory, Saskatoon, Sask. Issued as Paper No. 132 on the Industrial Utilization of Wastes and Surpluses, and N.R.C. No. 2668.

1/10 *N* iodine. Solid sodium bicarbonate was added to saturation (pH 8) and the bisulphite liberated from the bisulphite addition compound was titrated with exactly 1/10 *N* iodine. An analysis of 96% was obtained by this method on *n*-butyraldehyde redistilled at atmospheric pressure in air.

In some of the earlier work *n*-butyraldehyde was determined in aqueous solution by polarographic analysis. The diffusion current obtained (with a lithium chloride – lithium hydroxide carrier) was compared with standard samples. This method was convenient in some cases, but requires the nearly complete absence of all cations other than lithium. In addition it was found that the passage of nitrogen or hydrogen through the solution caused the *n*-butyraldehyde to disappear rapidly and it was necessary to clear the solution of dissolved oxygen before the addition of the sample.

The hydroxylamine hydrochloride method for aldehydes described by Bryant and Smith (3) was used for some of the samples in organic solvents when the bisulphite method was not suitable. All three methods were standardized against one another and good agreement was obtained.

Apparatus and Procedures

Solubilities and Distribution Coefficients

The mutual solubilities of water and *n*-butyraldehyde were determined by agitating stoppered flasks containing 50 ml. of each phase in a controlled temperature water bath until equilibrium was reached. The phases were allowed to separate completely and samples taken with pipettes or calibrated syringes. In cases where phase separation occurred in the pipette on heating or cooling to room temperature, specially calibrated pipettes were used for sampling. These were rinsed with appropriate solvent (water or butyraldehyde) to ensure complete transfer of the sample.

Distribution coefficients were determined in an analogous fashion.

Countercurrent Extraction

The two and 14-stage Scheibel columns used were those described in a previous paper (9). The same flow and interface controls were used.

Vapor–Liquid Equilibria

The apparatus used for the determination of vapor–liquid equilibrium compositions in the toluene–butyraldehyde system was substantially the same as that described by Othmer (4).

Chemical Losses of n-Butyraldehyde

Aqueous hydrochloric acid (100 ml.) and *n*-butyraldehyde (50 ml.) were shaken in a stoppered Erlenmeyer flask at controlled temperature as for solubility determinations. Samples of the carbonyl layer were analyzed for *n*-butyraldehyde at regular intervals by the bisulphite method described above. Experiments at 65° C. were made in a round bottomed 250 ml. flask fitted with an efficient reflux condenser and heated by a Glas Col mantle.

RESULTS AND DISCUSSION

The solubility of *n*-butyraldehyde reported in the literature (6) when this investigation was begun was known to be low from semiquantitative observations on phase volumes and specific gravities. Recently Smith and Bonner (7) have reported the solubility in water at 25° C. as 7.1 wt. % and the solubility of water in *n*-butyraldehyde at the same temperature as 3.0 wt. %. These latter values agree well with the results of this investigation, as shown in Figs. 1 and 2. The effect of both *n*-butyric acid and of the *n*-butyral of glycerol on the solubility of *n*-butyraldehyde in water was negligible, but both had an appreciable effect on the solubility of water in *n*-butyraldehyde (Figs. 2 and 3).

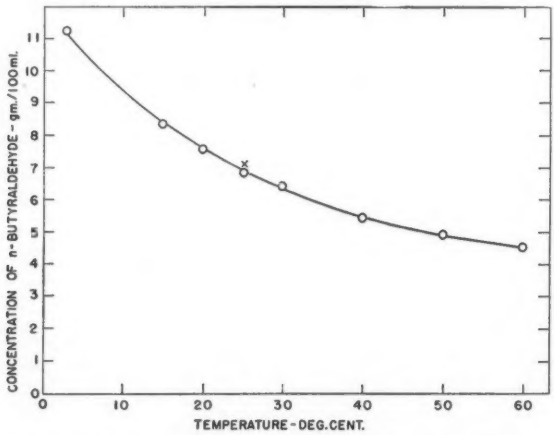

FIG. 1. Solubility of *n*-butyraldehyde in water. Effect of temperature. x—Smith and Bonner (7).

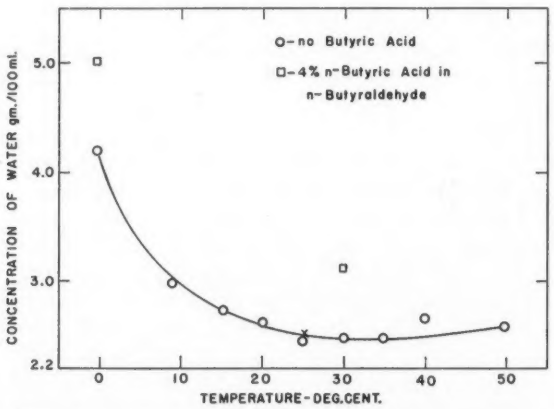

FIG. 2. Solubility of water in *n*-butyraldehyde. Effect of temperature and of *n*-butyric acid. x—Smith and Bonner (7).

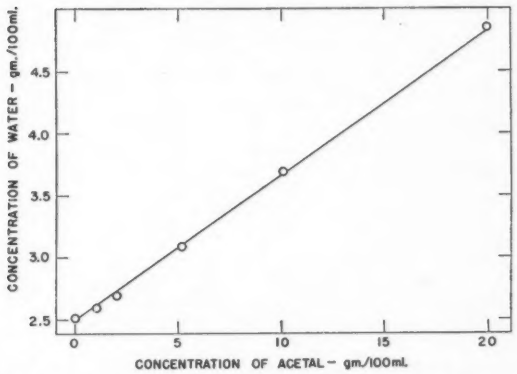

FIG. 3. Effect of the *n*-butyral of glycerol on the solubility of water in *n*-butyraldehyde at 30° C.

From Fig. 1 it is apparent that an appreciable amount of *n*-butyraldehyde will dissolve in the aqueous phase at temperatures used for the extraction of glycerol. For example, at 30° C. with a volume ratio of 0.5 (carbonyl phase water phase) about 16% of the *n*-butyraldehyde used will dissolve in the water. Experiments have shown that this may be recovered almost quantitatively by distillation (Table IV), particularly at low acid concentrations (Fig. 9) but a large amount of heat is required to raise the temperature of the dilute aqueous solution to the boiling point. Various solvents were therefore investigated to determine whether the recovery could not be carried out more efficiently by solvent extraction.

In Table I are shown the distribution coefficients for *n*-butyraldehyde between several solvents and water. Toluene is shown to be the most efficient extracting agent, and in addition has a suitable boiling point, is insoluble in water (1), and is inert under these conditions. The distribution curve for

TABLE I

DISTRIBUTION COEFFICIENTS FOR *n*-BUTYRALDEHYDE IN VARIOUS SOLVENT–WATER SYSTEMS AT 23° C.

Distribution coefficient		Distribution coefficient	
Toluene	12.83	Xylene	7.5
Benzene	10.4	Cymene	7.0
Carbon tetrachloride	9.9	Cyclohexane	3.8
Chlorobenzene	8.3	Skellysolve "S"	2.8

n-butyraldehyde between toluene and water is shown in detail in Fig. 4. It is interesting to note that temperature has no appreciable effect on the distribution, and that the distribution coefficient is constant within the error of determination over the whole concentration range investigated. The theo-

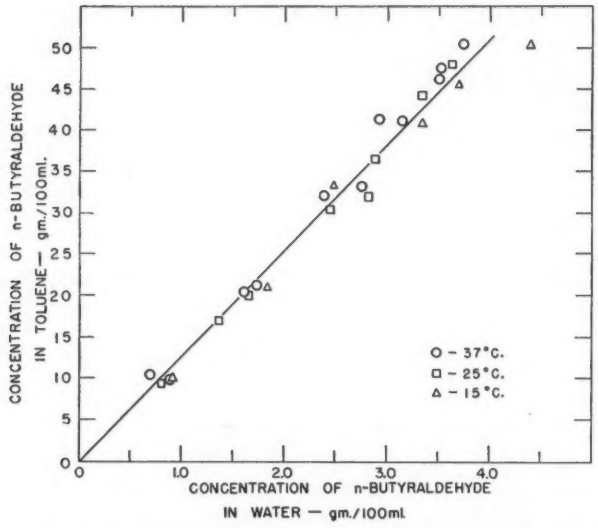

FIG. 4. Distribution of *n*-butyraldehyde between water and toluene.

retical extraction to be expected from a given number of extraction stages can be determined by simple mathematical calculation in this particular case (5), for either simple multiple contact or continuous countercurrent operation.

Experiments were made with two and 14-stage Scheibel columns (9) to determine their efficiencies for this extraction. It is shown in Table II that

TABLE II

TOLUENE EXTRACTION OF *n*-BUTYRALDEHYDE FROM WATER IN SCHEIBEL COLUMNS AT 30° C.

Stages	Ratio, water/toluene	Concentration of *n*-butyraldehyde (*M*)			
		Feed	Extract	Raffinate	Raffinate theor.*
2	3.0	0.75	1.9	0.09	0.04
2	9.34	0.6	3.18	0.21	0.13
14	10.0	0.57	3.37	0.16	0.03
14	10.1	0.56	3.4	0.17	0.03

**Calculated from Fig. 4.*

the stage efficiencies of these columns are low under these conditions, but that the greater part of the *n*-butyraldehyde can be recovered in a short column at high ratios of raffinate to solvent.

Since the *n*-butyraldehyde must be recovered from the relatively concentrated solution in toluene resulting from the solvent extraction, the vapor–liquid equilibrium curve for *n*-butyraldehyde and toluene was determined.

Calculations of column length and reflux ratio for a given separation can be made by the usual methods from the data shown in Fig. 5. No indication of azeotrope formation was found.

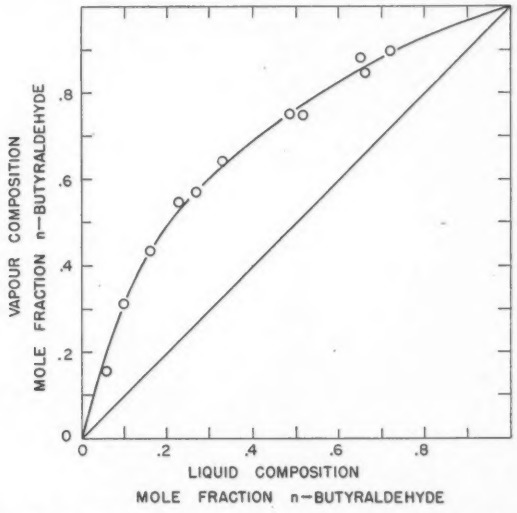

FIG. 5. Liquid–vapor equilibrium curve for toluene–*n*-butyraldehyde.

Although the solvent extraction with toluene was designed primarily to extract and recover the *n*-butyraldehyde, a certain amount of glycerol, in the form of the cyclic acetal, is also extracted. In Fig. 6 the distribution of

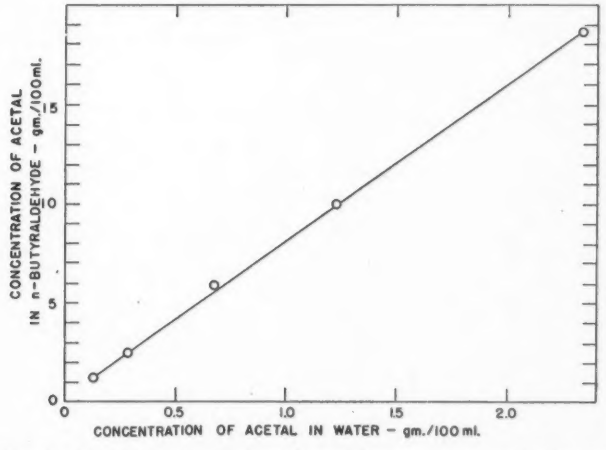

FIG. 6. Distribution of the *n*-butyral of glycerol between water and *n*-butyraldehyde at 30° C.

the acetal between the *n*-butyraldehyde and water layers in the initial extraction of glycerol is shown. When the aqueous phase is subsequently extracted with toluene the distribution curve shown in Fig. 7 indicates that some of the acetal will be extracted to the toluene layer along with the *n*-butyraldehyde. The distribution coefficient for this extraction is considerably less favorable than for the extraction of butyraldehyde, however, and recovery of the acetal would require distillation of the toluene.

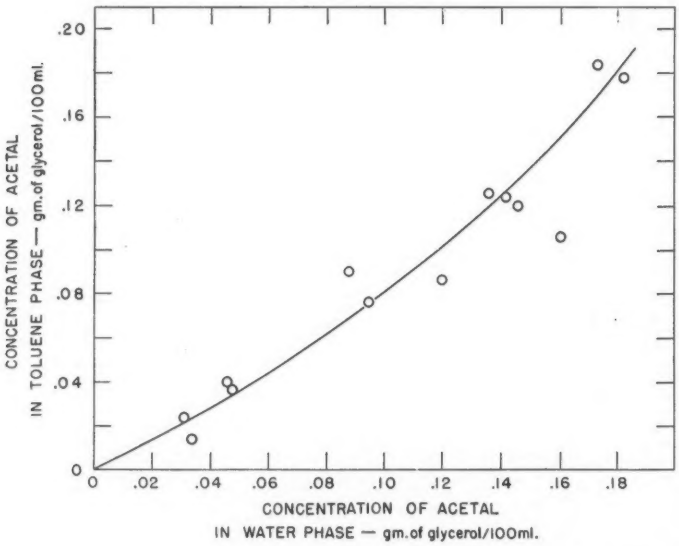

FIG. 7. Distribution of the *n*-butyral of glycerol between water and toluene at 30° C.

Chemical losses of *n*-butyraldehyde in the extraction process are more difficult to define than the physical. The products of chemical loss were assumed to be butyric acid and aldol type polymers. The butyric acid was isolated and identified in one experiment, but proved to be a minor component of the residue. The polymeric products were not identified, but were found to have no definite boiling point. The rate at which *n*-butyraldehyde disappeared from the carbonyl layer when agitated with either aqueous hydrochloric acid or a cation exchange resin in the acid form (Duolite C-3, 10-20 mesh prepared as in previous experiments (10)) was determined over a range of temperatures and acid concentrations. A typical loss curve is shown in Fig. 8, and it can be seen that the losses were neither uniform nor exactly reproducible. However, the rate of loss appears to increase approximately linearly with increased acidity (Fig. 9) and certainly increases very rapidly with increasing temperature (Table III).

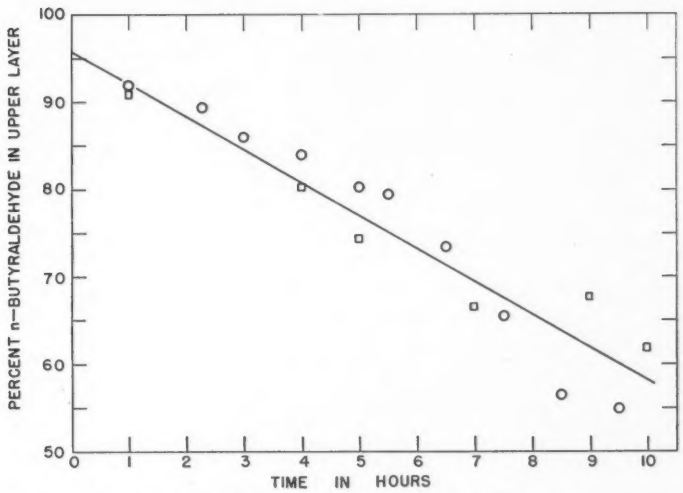

FIG. 8. Rate of loss of *n*-butyraldehyde at 40° C. in contact with 1.04 *N* hydrochloric acid. Duplicate experiments shown.

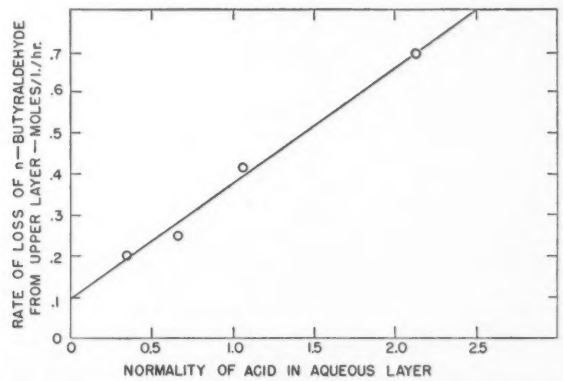

FIG. 9. Effect of acid concentration on the rate of loss of *n*-butyraldehyde at 65° C.

In Table III a comparison of rates of loss with hydrochloric acid and with the cation exchange resin is made. The amounts of resin were calculated to be the equivalent, for the extraction of glycerol, of two of the hydrochloric acid concentrations used. The addition of water in the experiments with the resin is shown to have no effect on the rate of loss, which indicates that no significant portion of the products of the reaction is extracted into the aqueous phase. The cation exchange resin is evidently a much better catalyst for the "polymerization" of *n*-butyraldehyde than for the formation of the cyclic acetal with glycerol.

TABLE III

EFFECT OF TEMPERATURE AND ACIDITY ON THE RATE OF DISAPPEARANCE OF *n*-BUTYRALDEHYDE

Acid conc. (*N*)	Ratio *n*-butyraldehyde to water	Temp., ° C.	Rate, moles/liter/hr.
2.91	0.5	40°	0.16
2.12	0.5	40°	0.13
		65°	0.69
1.04	0.5	40°	0.058
		65°	0.42
0.60	0.5	65°	0.25
0.32	0.5	65°	0.20
Duolite C-3 (10–20 mesh) gm./ml.			
0.033*	No water	65°	0.91***
	0.5	65°	0.90***
0.066**	No water	65°	1.13***
	0.5	65°	1.15***

**Equivalent to 0.3 N HCl as catalyst for the extraction of glycerol.*
***Equivalent to 0.6 N HCl as catalyst for the extraction of glycerol.*
****Rates are not linear. Loss at five hours was used for computing these values. Initial rates are approximately double these values.*

TABLE IV

THE RECOVERY OF *n*-BUTYRALDEHYDE BY DISTILLATION IN A TYPICAL BATCH EXTRACTION. TEN KGM. OF 4% AQUEOUS GLYCEROL SOLUTION (0. 378*N* WITH RESPECT TO HYDROCHLORIC ACID) AND 4. 10 KGM. (5 LITERS) OF *n*-BUTYRALDEHYDE REACTED FOR ONE HALF HOUR AT 30° C.

	Carbonyl layer		Aqueous layer	Total	
	Before hydrolysis	After hydrolysis		Kgm.	% Initial change
Amount of *n*-butyraldehyde originally in fraction (kgm.)	3.3	0.14	0.66	4.10	100
n-Butyraldehyde recovered from fraction (kgm.)	2.9	0.11	0.66	3.67	89.6
Time of distillation (hr.)	$3\frac{3}{4}$	$2\frac{1}{2}$	3		
Loss (kgm.)	0.4	0.03	0	0.43	10.4
Calculated maximum loss from Table III (kgm.)	0.29*	0.08	0.08	0.45	11.0

**Includes chemical loss during extraction.*

The actual recovery of *n*-butyraldehyde from a typical 15 liter extraction of glycerol is shown in Table IV. Both aqueous and carbonyl layers were stripped of butyraldehyde in an all glass still with a 6 ft., vacuum jacketed column packed with glass helices. The crude acetal was then hydrolyzed by refluxing with a small amount of cation exchange resin (Duolite C-3, 10-20 mesh, 33 gm. per liter) and the freed butyraldehyde continuously removed through a smaller packed column. For comparison, a rough estimate of losses on the basis of the data shown in Table III was made, assuming that the butyraldehyde was in contact with 0.378 *N* hydrochloric acid for the whole period of each distillation. Actual losses were approximately the same as these calculated maximum values.

Distillation of the carbonyl layer under reduced pressure (150. mm.) in a similar experiment did not reduce the chemical loss significantly. At this pressure the boiling point of *n*-butyraldehyde is 31° C. and of the azeotrope with water, 28° C., but with the long column used pot temperatures were very nearly as high as in the atmospheric distillation. From the data shown previously in Table III it would be expected that the loss of butyraldehyde could be reduced considerably by avoiding the local heating inherent in batch distillation in glass vessels.

REFERENCES

1. Bohon, R. L. and Clausen, W. F. J. Am. Chem. Soc. 73: 1571. 1951.
2. Bryant, W. M. D., Mitchell, J., Jr., and Smith, D. M. J. Am. Chem. Soc. 62: 3504. 1940.
3. Bryant, W. M. D. and Smith, D. M. J. Am. Chem. Soc. 57: 57. 1935.
4. Othmer, D. F. Ind. Eng. Chem. 35: 614. 1943.
5. Perry, J. M. (*Editor*) Chemical engineers' handbook. McGraw-Hill Book Co. Inc., New York, N.Y. 1941.
6. Seidell, A. Solubilities of organic compounds. D. Van Nostrand Co., New York, N.Y. 1941.
7. Smith, T. E. and Bonner, R. F. Ind. Eng. Chem. 43: 1169. 1951.
8. Tink, R. R. and Neish, A. C. Can. J. Technol. 29: 243. 1951.
9. Tink, R. R. and Roxburgh, J. M. Can. J. Technol. 29: 269. 1951.
10. Tink, R. R., Spencer, E. Y., and Roxburgh, J. M. Can. J. Technol. 29: 250. 1951.

CITRIC ACID FERMENTATION OF BEET MOLASSES BY *ASPERGILLUS NIGER* IN SUBMERGED CULTURE[1]

By Mary T. Clement

ABSTRACT

Optimum sugar concentration, pH, and ferrocyanide treatment were established for the production of citric acid by *Aspergillus niger* in submerged culture in beet molasses. Selected strains of *Aspergillus niger* from surface fermentations in ferrocyanide-treated beet molasses were used. In shake flask cultures under an oxygen atmosphere, the maximum yield of citric acid was equivalent to 52 gm. per 100 gm. of available sugar in a 10 day fermentation. In deep culture fermentations in Kluyver flasks aerated with air and oxygen, the maximum yield of citric acid was equivalent to 64 gm. per 100 gm. of available sugar in eight to nine days.

INTRODUCTION

Production of citric acid by molds was so greatly reduced in the transition from surface to submerged culture that for many years application of a submerged technique to this fermentation was considered impractical. Some progress has been made in determining the factors responsible for reduced yields in media of known composition (3, 4, 6, 8, 9, 10, 11, 12, 13, 14), but the use of crude carbohydrates has been limited by the necessity of removing inorganic constituents for controlled nutrition (4, 17). Recently good yields of citric acid in surface and submerged cultures have been reported from crude carbohydrates by addition of methanol (5). The addition of potassium ferrocyanide to crude beet molasses has proved an effective treatment for the production of citric acid by surface cultures on both laboratory and industrial scales (1, 2, 15), but no reference to a successful method for the production of citric acid from ferrocyanide-treated beet molasses in submerged culture could be found in the literature. The purpose of this work was to determine the feasibility of using submerged culture methods to produce citric acid from ferrocyanide-treated beet molasses in yields comparable to those obtained by surface methods.

MATERIALS AND METHODS

Strains

Cultures of *Aspergillus niger* were obtained from a collection of 200 strains surveyed for citric acid production in surface culture by Dr. G. A. Ledingham and his staff at the National Research Laboratories, Ottawa. The following strains were selected and maintained on molasses agar as described by Waller (15). The yields given were obtained from surface fermentation of ferrocyanide-treated beet molasses:

[1] *Manuscript received December 3, 1951.*
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as Paper No. 133 on the Uses of Plant Products and as N.R.C. No. 2674.

A. niger A-1-102—a local isolate from soil, yielding 40 to 50% of the titratable acids as citric acid with 50% conversion of sugar to citric acid; best of a number of strains tested for titratable acid production in submerged culture in synthetic medium by Karow's methods (3).

A. niger A-1-7—a local isolate from soil, yielding 90% of the titratable acids as citric acid with 50% conversion of sugar to citric acid.

A. niger A-1-215—a selected strain used in an industrial process in which citric acid was claimed to be the only acid end product and yields were equivalent to 70% sugar conversion (15).

Culture Methods

Chatham beet molasses containing approximately 50% sugar, and having a pH about 10, was diluted with distilled water to a sugar concentration of 16% and the pH was adjusted to the desired levels with 10 *N* sulphuric acid. A rise in pH occurred during sterilization, its magnitude increasing with increasing alkalinity of the medium. This resulted in difficulty in fixing the initial pH of the fermentation; hence both pre- and poststerilization levels are reported. Treatment of the medium with potassium ferrocyanide after sterilization without removal of the resulting precipitate proved effective and, for greater reproducibility of conditions, was used throughout this work. The precipitate cleared during the growth phase.

Shake flask fermentations were carried out in 1-liter Erlenmeyer flasks, each containing 200 ml. of medium. The inoculum was prepared from spores from a one-week-old molasses agar slant culture, suspended in 100 ml. of distilled water; 1 ml. of the suspension was used to inoculate each flask. The cultures were incubated at 28° C. on a rotary shaker having a horizontal radius of motion of $\frac{3}{4}$ in. and a speed of 104 r.p.m. After 48 hr. the cotton plugs of the flasks were replaced with aeration tubes connected in series of six to nine flasks. Humidified oxygen was passed through the flasks at the rate of 1 liter per minute, the inlet tubes being about one inch above the surface of the medium.

Deep culture fermentations were carried out in Kluyver flasks fitted with sintered glass aeration disks about 1 in. in diameter. In general, sugar concentration, pH, ferrocyanide treatment, and amount of inoculum were adopted from optima previously established in shake cultures. Maximum yields were obtained by the following procedure: 600 ml. of medium, having a sugar concentration of 12% and adjusted to pH 5.5, was sterilized in a Fernbach flask (1500 ml.) for 20 min. at 15 lb. pressure. The medium, poststerilization pH 5.8, was treated while hot with 0.6 or 0.8 gm. per liter of potassium ferrocyanide. A 5 ml. aliquot of dilute spore suspension prepared from a five day moist bran culture (yielding earlier and more abundant spores than molasses agar) was used to inoculate each lot. To avoid clogging the aeration disk of the Kluyver flask by germinating spores, the culture was incubated in the Fernbach flask on the shaker at 28° C. until the mycelia became visible as a light suspension in the medium, usually about 36 hr. after inoculation.

The young culture was then transferred to a sterile Kluyver flask and aerated with compressed air. Vigorous frothing that occurred at this stage was controlled by adding a few drops of oleic acid and by regulating the air supply. After initial frothing had subsided the air flow was carefully adjusted to the minimum necessary to keep the mycelia in circulation. When the period of maximum growth was over, usually about 72 to 96 hr. after inoculation, air was then replaced by oxygen and a sufficient rate maintained to provide steady circulation of the beadlike colonies throughout the medium.

Analytical Methods

When fermentations were terminated, the cultures were diluted two to three times the original volume with distilled water and steamed for 30 min. The mycelia were removed by filtration and the filtrates adjusted to suitable dilutions for analysis. Aliquots of the culture extracts were titrated with 0.25 *N* sodium hydroxide and total titratable acids expressed as citric acid. Citric acid was determined quantitatively as the monohydrate by the colorimetric method of Perlman, Lardy, and Johnson (7). Yields of citric acid were derived in the following manner:

$$\frac{\text{wt. citric acid}}{\text{wt. available sugar}} \times 100 = \text{percentage yield.}$$

RESULTS AND DISCUSSION

Shake Flask Fermentations

Ferrocyanide Treatment

Five concentrations of potassium ferrocyanide: 0, 0.3, 0.5, 0.7, 0.9 gm. per liter were tested with strain A-1-102 at pH levels of 6.5 and 8.0 (presterilization levels 6.0 and 7.0). Results (Table I) indicated that the optimal

TABLE I

EFFECTS OF FERROCYANIDE CONCENTRATION AT TWO pH LEVELS ON ACID PRODUCTION BY *A. niger* STRAIN A-1-102 IN SHAKE FLASKS
(12 days)

pH of medium	Potassium ferrocyanide, gm. per liter	Yields of titratable acids as citric on available sugar, %	Percentage of titratable acidity due to citric acid
6.5	0.0	15	27
(6.0)*	0.3	22	32
	0.5	46	40
	0.7	32	42
	0.9	48	40
8.0	0.0	23	23
(7.0)*	0.3	38	44
	0.5	30	30
	0.7	15	25
	0.9	16	21

**Presterilization pH.*

ferrocyanide concentration for citric acid production was dependent upon pH of the medium. At pH 8.0, highest yield was obtained from the lowest concentration of ferrocyanide, with a narrow optimal level about 0.3 gm. per liter and toxicity at the higher concentrations. At pH 6.5 increased tolerance to ferrocyanide was evident and the highest yield was obtained from the highest concentration with a stimulatory range from 0.5 to 0.9 gm. per liter. Addition of potassium dihydrogen phosphate (0.05%) appeared to counteract the toxic effects of the higher concentrations of ferrocyanide in alkaline media (pH 8.0) but yields did not exceed those from media with no additional phosphate at pH 6.5 to 6.8.

Maximum yields of titratable acids from A-1-102 were equivalent to about 50% on available sugar with citric acid comprising about 40% of the total.

Comparison of Strains

The low percentage of citric acid in the total acids produced by A-1-102 was due either to an inherent property of the strain or to displacement of citric acid to other organic acids by cultivation in a medium of high buffer content (16). In a preliminary comparison of A-1-102 with two strains, giving high percentages of citric acid in titratable acids in surface cultures, A-1-7 and A-1-215, approximately the same yields of citric acid (23%) were obtained from the three strains in submerged culture. However citric acid comprised about 70% of the total acids from A-1-7 and A-1-215 in contrast to 44% from A-1-102. Comparative response of the three strains to a range of initial pH levels was then determined in media at pH 6.0, 7.5, and 8.1 (presterilization pH 5.5, 6.5, 7.5), treated with 0.6 gm. of ferrocyanide per liter. Highest yields were obtained from A-1-215 and A-1-102 at pH 6.0 (Table II) with marked depression of acid production in alkaline media. Less variation in yields was shown by A-1-7 at different pH levels but the pro-

TABLE II

ACID PRODUCTION BY THREE STRAINS OF *A. niger* AT THREE pH LEVELS* IN SHAKE FLASKS
(13 days)

Strain	pH of medium	Yields of titratable acids as citric on available sugar, %	Yields of citric acid on available sugar, %	Percentage of titratable acidity due to citric acid
A-1-102	6.0	53	24	44
	7.5	16	2	12
	8.1	18	3	17
A-1-215	6.0	46	31	67
	7.5	20	6	33
	8.1	23	9	38
A-1-7	6.0	23	11	48
	7.5	20	13	65
	8.1	26	15	57

*0.6 gm. of ferrocyanide per liter.

The young culture was then transferred to a sterile Kluyver flask and aerated with compressed air. Vigorous frothing that occurred at this stage was controlled by adding a few drops of oleic acid and by regulating the air supply. After initial frothing had subsided the air flow was carefully adjusted to the minimum necessary to keep the mycelia in circulation. When the period of maximum growth was over, usually about 72 to 96 hr. after inoculation, air was then replaced by oxygen and a sufficient rate maintained to provide steady circulation of the beadlike colonies throughout the medium.

Analytical Methods

When fermentations were terminated, the cultures were diluted two to three times the original volume with distilled water and steamed for 30 min. The mycelia were removed by filtration and the filtrates adjusted to suitable dilutions for analysis. Aliquots of the culture extracts were titrated with 0.25 *N* sodium hydroxide and total titratable acids expressed as citric acid. Citric acid was determined quantitatively as the monohydrate by the colorimetric method of Perlman, Lardy, and Johnson (7). Yields of citric acid were derived in the following manner:

$$\frac{\text{wt. citric acid}}{\text{wt. available sugar}} \times 100 = \text{percentage yield.}$$

RESULTS AND DISCUSSION

Shake Flask Fermentations

Ferrocyanide Treatment

Five concentrations of potassium ferrocyanide: 0, 0.3, 0.5, 0.7, 0.9 gm. per liter were tested with strain A-1-102 at pH levels of 6.5 and 8.0 (presterilization levels 6.0 and 7.0). Results (Table I) indicated that the optimal

TABLE I

EFFECTS OF FERROCYANIDE CONCENTRATION AT TWO pH LEVELS ON ACID PRODUCTION BY *A. niger* STRAIN A-1-102 IN SHAKE FLASKS
(12 days)

pH of medium	Potassium ferrocyanide, gm. per liter	Yields of titratable acids as citric on available sugar, %	Percentage of titratable acidity due to citric acid
6.5	0.0	15	27
(6.0)*	0.3	22	32
	0.5	46	40
	0.7	32	42
	0.9	48	40
8.0	0.0	23	23
(7.0)*	0.3	38	44
	0.5	30	30
	0.7	15	25
	0.9	16	21

**Presterilization pH.*

ferrocyanide concentration for citric acid production was dependent upon pH of the medium. At pH 8.0, highest yield was obtained from the lowest concentration of ferrocyanide, with a narrow optimal level about 0.3 gm. per liter and toxicity at the higher concentrations. At pH 6.5 increased tolerance to ferrocyanide was evident and the highest yield was obtained from the highest concentration with a stimulatory range from 0.5 to 0.9 gm. per liter. Addition of potassium dihydrogen phosphate (0.05%) appeared to counteract the toxic effects of the higher concentrations of ferrocyanide in alkaline media (pH 8.0) but yields did not exceed those from media with no additional phosphate at pH 6.5 to 6.8.

Maximum yields of titratable acids from A-1-102 were equivalent to about 50% on available sugar with citric acid comprising about 40% of the total.

Comparison of Strains

The low percentage of citric acid in the total acids produced by A-1-102 was due either to an inherent property of the strain or to displacement of citric acid to other organic acids by cultivation in a medium of high buffer content (16). In a preliminary comparison of A-1-102 with two strains, giving high percentages of citric acid in titratable acids in surface cultures, A-1-7 and A-1-215, approximately the same yields of citric acid (23%) were obtained from the three strains in submerged culture. However citric acid comprised about 70% of the total acids from A-1-7 and A-1-215 in contrast to 44% from A-1-102. Comparative response of the three strains to a range of initial pH levels was then determined in media at pH 6.0, 7.5, and 8.1 (presterilization pH 5.5, 6.5, 7.5), treated with 0.6 gm. of ferrocyanide per liter. Highest yields were obtained from A-1-215 and A-1-102 at pH 6.0 (Table II) with marked depression of acid production in alkaline media. Less variation in yields was shown by A-1-7 at different pH levels but the pro-

TABLE II

ACID PRODUCTION BY THREE STRAINS OF *A. niger* AT THREE pH LEVELS* IN SHAKE FLASKS
(13 days)

Strain	pH of medium	Yields of titratable acids as citric on available sugar, %	Yields of citric acid on available sugar, %	Percentage of titratable acidity due to citric acid
A-1-102	6.0	53	24	44
	7.5	16	2	12
	8.1	18	3	17
A-1-215	6.0	46	31	67
	7.5	20	6	33
	8.1	23	9	38
A-1-7	6.0	23	11	48
	7.5	20	13	65
	8.1	26	15	57

**0.6 gm. of ferrocyanide per liter.*

portion of citric acid to the total titratable acids was higher in alkaline media. Low proportions of citric acid to total acids characteristic of A-1-102 confirmed the unsuitability of this strain for citric acid production, and its use was discontinued.

In further studies on the requirements for high yields, the following data were obtained from A-1-7 in a fermentation at pH 7.8, prolonged to 18 days:

Potassium ferrocyanide, gm. per liter	Yields of citric acid, %	Percentage of titratable acidity due to citric acid
0.4	18	50
0.8	35	84
1.2	18	69

At the optimum ferrocyanide concentration, the percentage of titratable acidity due to citric acid from strain A-1-7 approached the maximum obtained in surface fermentations, but the fermentation time was unduly long.

Strain A-1-215 gave the highest yield (Table II) at an acid pH—an advantage because of greater stability of pH, increased tolerance to ferrocyanide, and value of low initial pH in controlling bacterial contamination. The lower limits of pH for growth and acid production for A-1-215 were therefore determined in media at pH levels of 4.0, 4.8, and 5.8, treated with 0.3, 0.6, 0.9 gm. of ferrocyanide per liter. No growth occurred at pH 4.0. Most satisfactory yields were obtained at pH 5.8 (Table III) with the highest

TABLE III

YIELDS FROM STRAIN A-1-215 IN ACID MEDIA AT THREE FERROCYANIDE CONCENTRATIONS IN SHAKE FLASKS

(12 days)

pH of medium	Potassium ferrocyanide, gm. per liter	Titratable acids as citric on available sugar, %	Citric acid on available sugar, %	Percentage of titratable acidity due to citric acid
4.85	0.3	37	23	62
(4.8)*	0.6	34	19	57
	0.9	26	13	50
5.8	0.3	38	23	62
(5.5)*	0.6	42	34	80
	0.9	45	34	75

**Presterilization pH.*

proportion of citric acid in the total acids, 80%, at a concentration of 0.6 gm. of ferrocyanide. However total titratable acid yields were only 42 to 45% on available sugar.

Effects of Molasses Concentration on Efficiency of Citric Acid Production

The effects of various concentrations of molasses on yields were tested with strain A-1-215 in molasses media diluted to concentrations of 4, 8, 12, 16, and

20% sugar treated with 0.15, 0.3, 0.45, 0.6, 0.75 gm. of ferrocyanide per liter respectively, at a poststerilization pH of 5.8. Results (Table IV) showed that in a 10 day fermentation more efficient sugar conversion was obtained at 8 and 12% concentrations than at the 16% concentration previously employed. Approximately the same quantities (i.e. gm. per 100 ml. of

TABLE IV

EFFECTS OF VARIOUS MOLASSES CONCENTRATIONS ON YIELDS OF CITRIC ACID BY A-1-215 IN SHAKE FLASKS

(10 days)

Concentration of sugar in medium, %	Yields of titratable acids as citric on available sugar, %	Yields of citric acid on available sugar, %	Percentage of titratable acidity due to citric acid
4	45	22	48
8	62	52	83
12	58	52	90
16	48	40	83
20	40	31	78

STATE COLLEGE OF AGRICULTURE LIBRARY MAY 12 1952 MANHATTAN KANSAS

medium) of citric acid were produced from concentrations of 12, 16, and 20% sugar, with the resulting percentage yields showing a relative decrease in values. In a similar experiment extended to 14 days, acid production from 12% sugar reached the peak and tapered off at the 10th day. Although acid production continued at sugar concentrations of 16 and 20%, further gains did not justify the prolonged fermentation.

Yields of citric acid equivalent to 52% from several fermentations appeared to be the maximum obtainable under given conditions of agitation and oxygenation. Without proceeding further with experiments in shake cultures, studies were undertaken with deep cultures under conditions more comparable to those found in vat fermentations.

Deep Culture Fermentations

Regulation of growth to obtain discrete bead-type colonies readily circulated by the oxygen stream was essential to good yields in deep cultures. Fermentations in which the mycelia developed as a thick sludge could not be adequately aerated and were usually terminated before completion owing to blocking of the aeration disk. Conditions governing desirable colony formation were difficult to determine, but it was noted that the rate of aeration of the young mycelia in the Kluyver flask had a marked effect upon the number and form of the colonies. By restricting aeration to the minimum necessary to keep the mycelia from settling out and clogging the aeration disk, improvement in colony conformation was obtained and the frequency of sludgy fermentations considerably reduced.

Over 50 fermentations were set up in the development of the deep culture method described in a preceding section. Maximum yields are shown in the following table:

Strain	Potassium ferrocyanide, gm. per liter	Duration (days)	Titratable acids as citric, %	Citric acid, %
A-1-215	0.8	9	75	64
A-1-215	0.8	8	74	60
A-1-7	0.6	12	63	55

The peak in acid production by A-1-215 in these fermentations was approached at seven days. Strain A-1-7 showed improved activity under deep culture conditions and in some runs yields were comparable to A-1-215 but the fermentations were of longer duration.

While these results show the possibility of good yields from deep cultures in ferrocyanide-treated molasses, uniform yields were not obtained under the conditions described. Variations were attributed partly to inadequate control of aeration and the use of a substrate not constant in composition. However a better understanding of the factors influencing the development of mycelia in deep culture would be necessary in order to achieve controlled growth essential to uniform yields. Further study of this fermentation has been undertaken by other investigators in these laboratories.

REFERENCES

1. BERNHAUER, K., RAUCH, J., and GROSS, G. Biochem. Z. 319: 499-514. 1949.
2. GERHARDT, P., DORRELL, W. W., and BALDWIN, I. L. J. Bact. 52: 555-564. 1946.
3. KAROW, E. O. Ph.D. thesis, Rutgers University. 1942.
4. KAROW, E. O. and WAKSMAN, S. A. Ind. Eng. Chem. 39: 821-825. 1947.
5. MOYER, A. J. Bact. Proc. Soc. Am. Bact. 51st general meeting. 1951.
6. PERLMAN, D. Econ. Botan. 3: 360-374. 1949.
7. PERLMAN, D., LARDY, H. A., and JOHNSON, M. J. Ind. Eng. Chem., Anal. Ed. 16: 515-516. 1944.
8. PERQUIN, L. H. C. Dissertation, Delft. 1938.
9. SCHWEIGER, L. B. and SNELL, R. L. U.S. Patent 2, 476, 159. 1949.
10. SHU, P. and JOHNSON, M. J. J. Bact. 54: 161-167. 1947.
11. SHU, P. and JOHNSON, M. J. J. Bact. 56: 576-585. 1948.
12. SHU, P. and JOHNSON, M. J. Ind. Eng. Chem. 40: 1202-1205. 1948.
13. SZÜCS, J. U.S. Patent 2, 353, 771. 1944.
14. WAKSMAN, S. A. and KAROW, E. O. U.S. Patent 2, 394, 031. 1946.
15. WALLER, C. J. British Intelligence Objectives Subcommittee, BIOS Final Rept. No 489, Item No. 22. 1946.
16. WELLS, P. A. and WARD, G. E. Ind. Eng. Chem. 31: 172-177. 1939.
17. WOODWARD, J. C., SNELL, R. L., and NICHOLLS, R. S. U.S. Patent 2, 492,673. 1949.

thermistor element designated No. 67. Resistance pressure curves were obtained for No. 64 for constant temperatures of 0° C., 25° C., and 50° C. for pressures up to 2000 lb. per in^2. Similar curves were obtained for No. 67 for temperatures of 25° C. and 50° C. These curves are shown in Figs. 2 to 6.

APPARATUS

The thermistor under test was placed inside a small steel bottle fitted with a valve. One thermistor lead was grounded to the bottle while the other passed through an insulated plug on the valve. A Wheatstone bridge circuit measured the thermistor resistances accurately to one ohm. The pressure was supplied by a high pressure cylinder of nitrogen. The steel bottle was immersed in a constant temperature water bath whose temperature was controlled to within 0.1° C. for the preliminary tests and to within 0.01° C. for the later tests. With the temperature held constant the pressure was varied

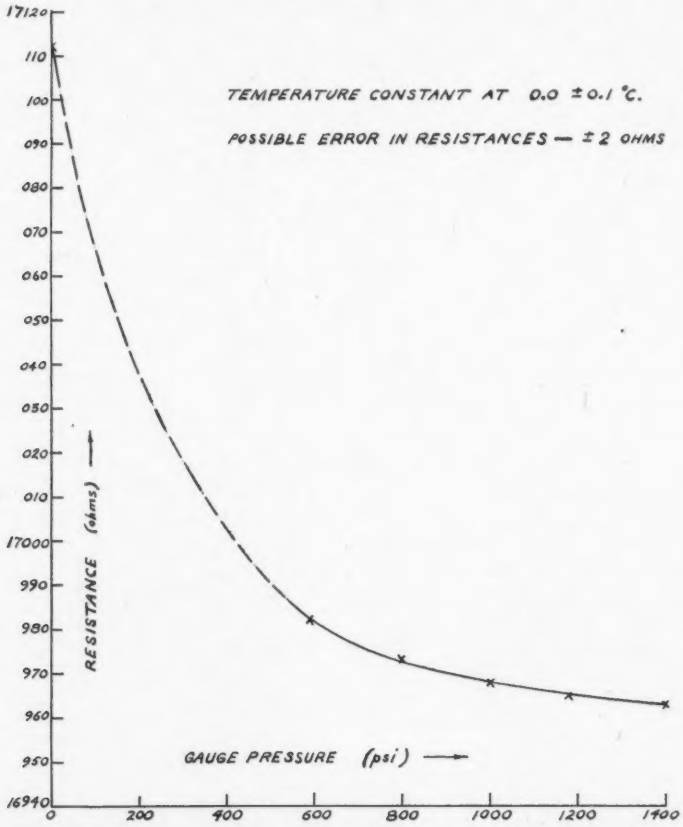

FIG. 2. Resistance–pressure curve, thermistor No. 64, temperature 0.0° C.

in steps of 100 to 200 lb. per in.^{2} from 0 to nearly 2000 lb. per in.^{2} above atmospheric pressure and corresponding thermistor resistances were measured. The possible error in the resistance measured depended on the temperature fluctuation of the bath. The bath temperature and possible error in resistances for each test are recorded on the curves.

RESULTS

Figs. 2 to 6 show that the thermistors tested experience a decrease in resistance with increased pressure at constant temperatures. In every case

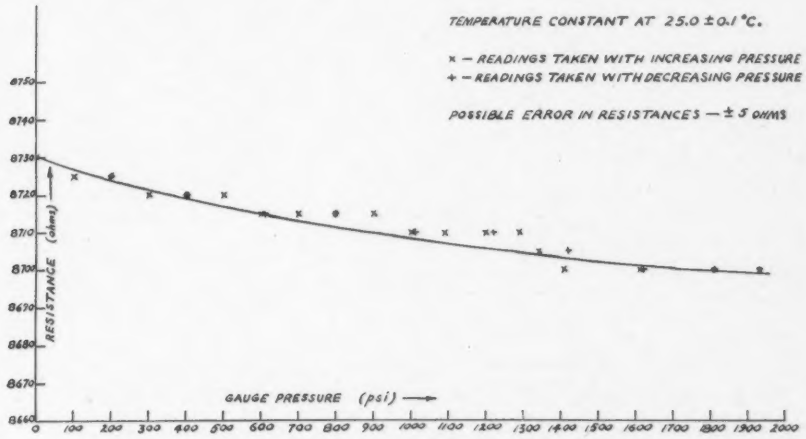

FIG. 3. Resistance–pressure curve, thermistor No. 64, temperature 25.0° C.

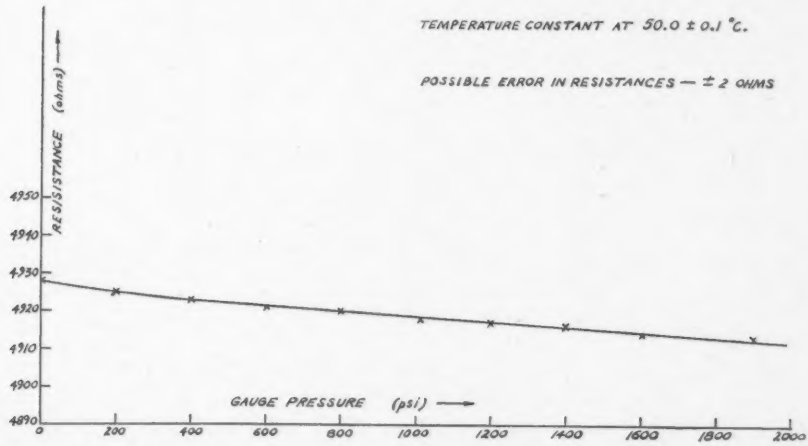

FIG. 4. Resistance–pressure curve, thermistor No. 64, temperature 50.0° C.

smooth curves can be drawn through the experimental points to within the experimental error. Most of the decrease in resistance occurs within the first few hundred pounds per square inch pressure. The change gradually becomes less with increased pressure until, near 2000 lb. per in.^{2}, there is almost no further decrease in resistance at temperatures below 25° C. At 50° C. and 1800 lb. per in.^{2} pressure there is still an apparent change of resistance with pressure of 1 ohm per 100 lb. per in.^{2} (Fig. 6). Such a change of

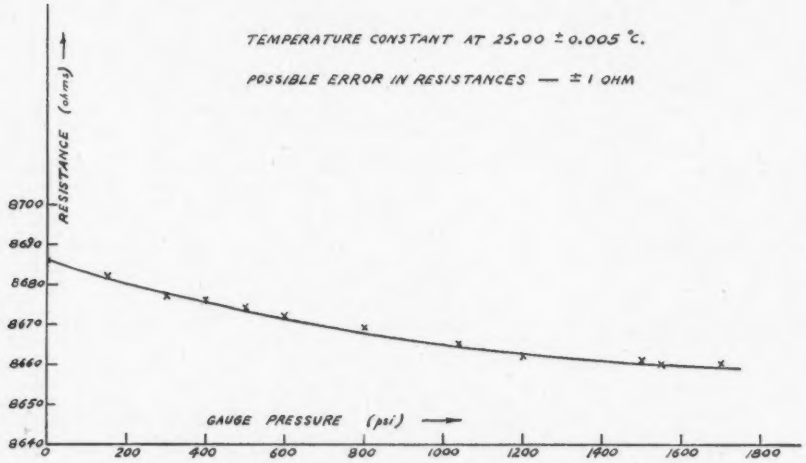

FIG. 5. Resistance–pressure curve, thermistor No. 67, temperature 25.00° C.

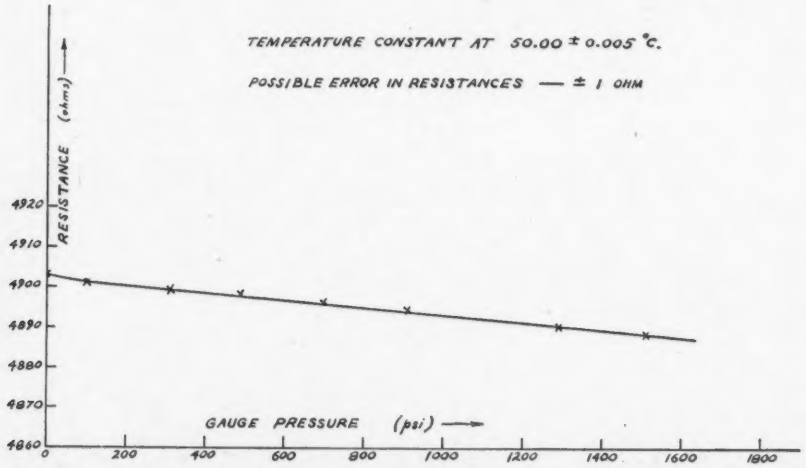

FIG. 6. Resistance–pressure curve, thermistor No. 67, temperature 50.00° C.

resistance corresponds to a change of only 0.005° C. (Fig. 7) and is therefore negligible for most practical work. The pressure coefficient of resistance is not constant but varies with pressure. Above 2000 lb. per in.^{2} the pressure coefficient apparently approaches a zero value.

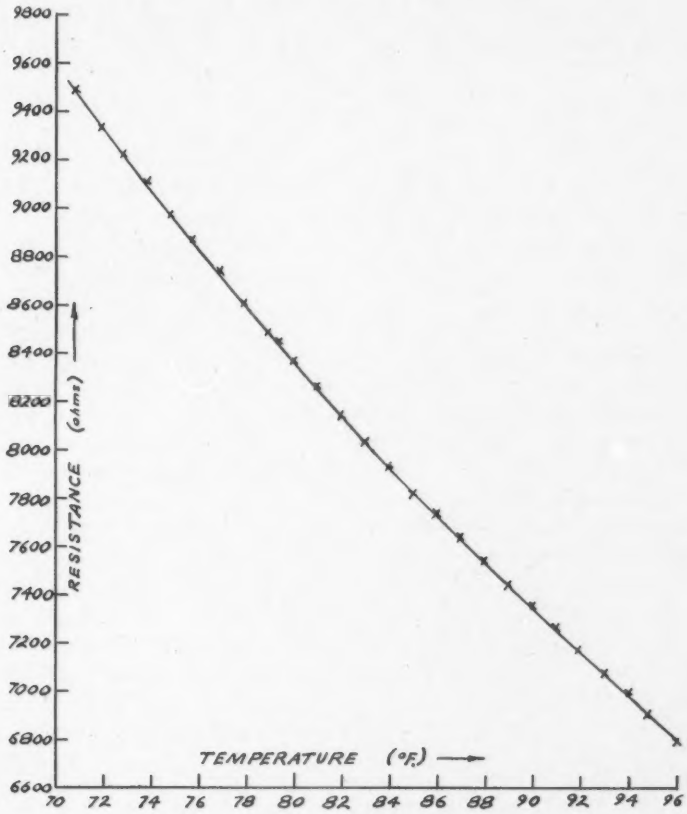

FIG. 7. Calibration curve for No. 65.

The pressure coefficient of resistance is defined by the relation

$$\lambda_p = \frac{1}{R_0}\left(\frac{dR}{dp}\right)_p$$

where λ_p = pressure coefficient of resistance at pressure p
R_0 = the resistance at atmospheric pressure p_0 in ohms
$(dR/dp)_p$ = the slope of the resistance–pressure curve at the chosen pressure p.

Slopes were taken for each experimental curve at 0 lb. per in.^{2} and 1000 lb. per in.^{2} and corresponding pressure coefficients were calculated. It was found

that the pressure coefficient at any one pressure was very nearly the same for the different temperatures involved. The average value of λ_0 was -3.0×10^{-6} per lb. per in.^{2} while that of λ_{1000} was -1.5×10^{-6} per lb. per in.^{2} Near 2000 lb. per in.^{2} the pressure coefficient approached a zero value. The possible error in drawing the curves and taking slopes was quite high and so it was possible that any one particular value of λ_p might have been in error up to about $\pm$ 15%. All the values of λ_p at any one pressure showed a reasonable agreement within this possible error, and it appeared that λ_p for any one pressure was constant for any temperature within the range tested. More detailed work must be done to verify this conclusion.

From the calibration of one of these thermistor thermometers used to measure temperatures to $\pm$ 0.01° C., it has been found that a change in temperature of 0.1° C. produced a change in resistance of 11.5 ohms at 50° C. At 25° C. the change was 21.1 ohms per 0.1° C. change, and at 0° C. the change was 48.3 ohms for 0.1° C. change. Since the thermistors tested were similar to this one, it can be seen from the resistance–pressure curves that the decrease in resistance for the maximum pressure used at 0° C. corresponds to a temperature rise of just over 0.3° C. At 25° C. and 50° C. the decrease in resistance corresponds to a temperature rise of less than 0.2° C. Hence for temperature measurements less accurate than $\pm$ 0.1° C. the effect of pressure could be neglected. For temperature measurements accurate to $\pm$ 0.1° C. the change in resistance due to pressure is within the range of error of temperature measurement except for temperatures near 0° C. For temperature measurements more accurate than, and including, 0.1° C. a correction should be made for the effect of pressure.

The resistance–temperature calibration curve for thermistor thermometers is usually obtained at atmospheric pressure. If such a thermometer is used to measure temperatures while under pressure, its measured resistance would be lower than at atmospheric pressure by an amount dR. If the excess pressure p and the resistance–pressure curves for the thermistor thermometer are known, then the value of dR may be determined and the original resistance reading corrected.

STUDIES ON THE TREATMENT OF CONTAMINATED COMBS AND HONEY WITH HIGH VELOCITY ELECTRONS[1]

By H. Katznelson,[2] C. A. Jamieson,[3] E. J. Lawton[4] and W. D. Bellamy[4]

ABSTRACT

High velocity electrons are effective in killing *Bacillus larvae* spores in thin sections of comb containing American foulbrood scale and in honey. The lethal dose for an initial concentration of 4×10^6 spores per ml. is in the range of 2 to 4×10^6 equivalent roentgen, a value that is higher than that required for other sporeformers, such as *Bacillus subtilis*. Honey of the initial concentration irradiated with sublethal doses 1×10^6 and 2×10^6 r. did *not* cause AFB disease when fed to healthy colonies of bees, whereas the unirradiated honey produced extensive disease within one month. Honey contaminated naturally with osmophilic yeasts (initial concentration 2040 per ml.) was completely sterilized by an irradiation dose of 0.5×10^6 r. Honey contaminated with six strains of sugar-tolerant yeasts (initial concentration 128,000 per ml.) required a dose of 1 to 2×10^6 r. for complete sterility. There was definite indication that there were at least two yeast types of different radiosensitivity; one being three to four times more resistant than the other. The more resistant type is about as radioresistant as AFB spores. There were no appreciable organoleptic changes in honey exposed to an irradiation dose as high as 5×10^6 r. Doses as high as 5×10^6 r. do not prevent the reuse of the comb by the bees.

INTRODUCTION

Bacillus larvae, the cause of American foulbrood (AFB) of the honey bee is of concern to beekeepers in every part of the world. AFB disease can spread either through a reuse of the infected comb or feeding of infected honey. This prevents a safe reuse of the comb and honey without possible loss of the entire bee colony. The standard method of control is burning of the infected material which constitutes a large annual loss to the industry. Methods of control through the use of sulfa drugs have met with moderate success (8, 9). Since it is possible to destroy microorganisms with ionizing irradiation (2, 13), such as produced by high energy electrons or cathode rays, it was decided to explore the effectiveness of these as a means of killing AFB spores. The use of cathode rays, rather than X rays, is preferable because the over-all efficiency of the cathode-ray system is many times that of the X-ray system, so that the large doses required can be accumulated in a matter of seconds rather than minutes or hours as would be required for X rays. The associated problem of honey contamination with such spores, or osmophilic yeasts which are responsible for fermentation and spoilage of honey, was examined at the same time.

METHOD

A resonance transformer type cathode-ray unit provided a source of high velocity electrons with which a dose was accumulated at the rate of approxi-

[1] *Manuscript received December 10, 1951.*
Joint contribution from the Department of Agriculture, Ottawa, Canada, and General Electric Company, Schenectady, N.Y. (No. 331, Division of Bacteriology and Dairy Research.)
[2] *Division of Bacteriology and Dairy Research, Science Service.*
[3] *Bee Division, Experimental Farms Service.*
[4] *General Electric Company.*

mately 0.14×10^6 equivalent roentgens per second at 10 cm. from the window (1, 11, 12). Irradiation in all cases was with electrons of 800 kv. (peak) energy. This will provide a fairly uniform dose to material not thicker than 3 mm. of water equivalent when irradiated from both sides. Samples of honey and comb were sealed into $2\frac{1}{2}$ in. diameter containers of 0.003 in. thick polyethylene. Samples were irradiated while in the container and were not removed until the biological tests were run. In the case of honey, a 2 gm. sample spread to a thin layer within the container resulted in a layer thickness (0.1 gm. per cm.^{2}) easily penetrated by the electrons and throughout which a fairly uniform irradiation dose was realized when the layer was irradiated from one side. Normal used comb containing capped and partially filled cells is too thick, in most cases, for penetration by 800 kiloelectron volts. Therefore, in those experiments where contaminated combs were irradiated, the comb was sliced into thin sections 3 mm. thick and irradiated from both sides to ensure uniform dose throughout the thickest section. Not all of the section was of uniform 3 mm. thickness, so that only those parts of the section that were of this thickness received a uniform dose throughout when irradiated from both sides. The dose, therefore, is variable over the comb section and varied as much as a factor of two between the thin and the thick parts. The doses given for the comb are the minimum values which would correspond to the thickest portion of the section. Irradiation dose, expressed in equivalent roentgens, was determined from ionization measurements with a specially constructed ionization chamber.

EXPERIMENTAL RESULTS

AFB in Comb Slices

Thin comb sections were irradiated at different dose levels and then examined for viable *B. larvae* spores. Three separate scales were removed aseptically from each sample into sterile water in Petri plates and macerated. Two loopfuls (3 mm. loop) were transferred from each scale to yeast–carrot semisolid agar medium (14), the cultures were incubated at 37° C., and examined periodically for *B. larvae* cells, yeasts, and for nitrate reduction. The material remaining in the Petri plates was allowed to dry and tested for enzyme activity by the milk coagulation test (5, 10). The results of these preliminary tests are shown in Table I. Complete destruction of AFB spores and elimination of yeasts were achieved in sections receiving an irradiation dose of 1×10^6 r. or greater. Nitrite tests for *B. larvae* (15) paralleled the results of microscopic examination of the cultures for this organism. The enzymes in AFB scale concerned with milk coagulation and digestion (5, 6, 10) were not deactivated at 2×10^6 r. Enzymes have been reported to be more radioresistant (7, 13) than microorganisms.

Further studies were made using thin sections of comb, to test the effect of incubating medium. In addition, AFB scale was placed on sterile wood blocks, covered with a thin layer of sterile wax, and irradiated with 1.0 and 2.0×10^6 r. Tests for spore viability were carried out as described except

that two semisolid media were used and four scales were removed from every treated sample. The media were identical except that one contained 1% soluble starch (3, 4); the remaining constituents were: peptone, 10.0 gm.; yeast extract, 10.0 gm.; glucose, 5.0 gm.; agar, 3.0 gm.; potassium nitrate, trace; and distilled water, 1 liter. Representative data for the thin comb are

TABLE I

EFFECT OF CATHODE RAYS ON AFB SCALE IN SECTIONED COMB*

Dose × 10^6 r.	*B. larvae* cells	Yeasts	Nitrite production	Proteolytic enzyme
0	3	3	3	3
0.1	3	3	3	3
0.2	3	3	3	3
0.5	1	0	1	3
1.0	0	0	0	3
2.0	0	0	0	3

**After 14 days' incubation, expressed as the number of scales out of three showing positive results.*

listed in Table II. The results appear to corroborate those in Table I and indicate no significant difference between the two media, and that the lethal dose is in the range 1 to 2 × 10^6 r. This dose did not deactivate the enzymes.

The AFB scale on wood blocks was inactivated by 1 and 2 × 10^6 r., whereas untreated controls gave abundant growth and nitrite liberation in the starch medium.

TABLE II

COMPARISON OF COMPOSITION OF GROWTH MEDIUM ON SURVIVAL OF AFB IN SECTIONED COMB

Dose × 10^6 r.	*B. larvae*	Nitrite production	Proteolytic enzyme
*Medium 1**			
0	4	4	4
0.5	1	1	4
1.0	1	1	4
2.0	0	0	4
Medium 1 + starch			
0	4	4	4
0.5	1	1	4
1.0	1	3	4
2.0	0	0	4

**Composition given in text.*

NOTE: *Microscopic examination at one week.*
Nitrite test at three weeks.
Results expressed as number of scales out of four showing positive results.

Whole comb was exposed to a high dose, 5×10^6 r., to determine whether any undesirable effects were produced that would prevent the bees reusing the comb. When this comb was incorporated in a frame and placed in a colony no AFB disease developed; the scale was removed by the bees and the queen laid eggs in the cells, thus demonstrating that the treated comb had no properties objectionable to the queen.

AFB Spores in Honey

Liquid honey was inoculated with spores from scale and 2-gm. samples irradiated with doses from 0.025 to 2.0×10^6 r. One-gram amounts were then removed as aseptically as possible into test tubes with 9.0 ml. water, shaken thoroughly, and decimal dilutions made. Each dilution was plated with the starch medium and plates counted after four-five days at 37° C.

TABLE III

KILLING EFFECT OF HVE ON AMERICAN FOULBROOD SPORES IN HONEY

Dose $\times 10^6$ r.	Number	% Survivors
Experiment 1		
Control *A*	2,800,000	100.0
Control *B*	4,200,000	
0.025	500,000	14.0
0.05	400,000	11.4
0.1	390,000	11.1
0.25	220,000	6.3
0.5	35,000	1.0
0.75	23,000	0.66
1.0	28,000	0.8
2.0	190	0.0054
Experiment 2		
Control *A*	4,200,000	100.0
Control *B*	4,200,000	
0.1 *A*	510,000	13.0
0.1 *B*	590,000	
0.25 *A*	35,000	1.4
0.25 *B*	81,000	
0.5 *A*	1400	0.4
0.5 *B*	1990	
1.0 *A*	10	0.035
1.0 *B*	280	
2.0 *A*	10	0.0024
2.0 *B*	10	
4.0 *A*	0	0
4.0 *B*	0	

After counting, representative colonies were picked into semisolid agar and the culture examined microscopically and tested for nitrite. The data for two separate experiments are given in Table III and shown in Fig. 1 as Curves 1 and 2. Although a dose as low as 0.025×10^6 r. caused a marked reduction in the number of viable spores, there were still some that resisted 2×10^6. The result of Experiment 2 indicates that there were no survivors at 4×10^6 r. The lethal dose, therefore, for this organism in a concentration of 4.2×10^6 per ml. is between 2 and 4×10^6 r. Under the condition described, we have found these spores are more resistant than those of *B. subtilis*, which in equal

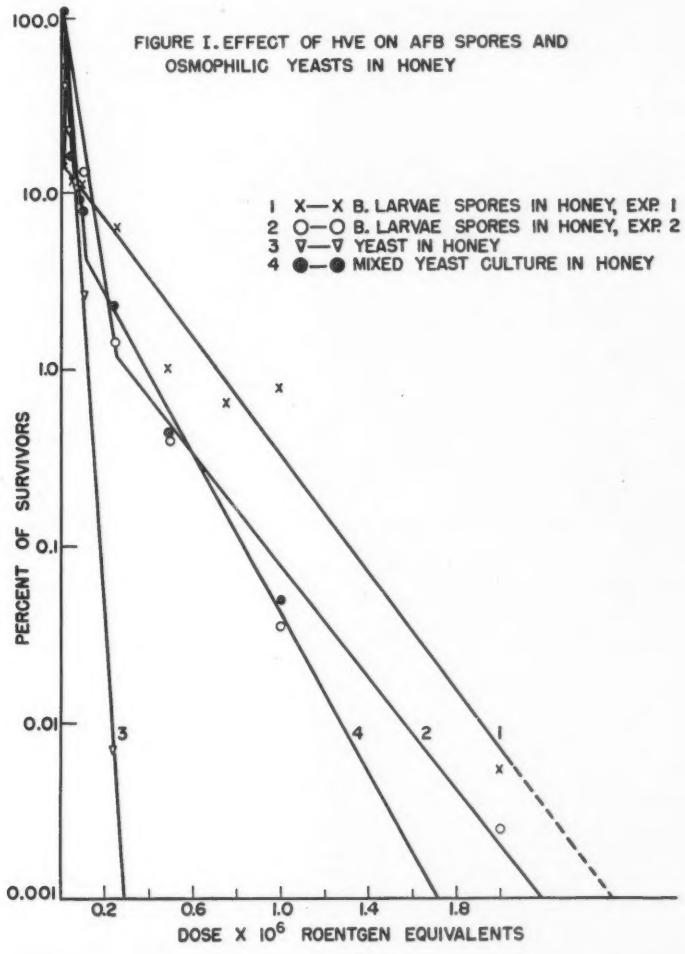

FIG. 1.

concentrations are completely sterilized by $0.6–0.7 \times 10^6$ r. Spores of *Clostridium sporogenes* are also more sensitive to irradiation than are spores of *B. larvae.* Dunn *et al.* (2) report destruction of *B. cereus* (28,000,000 per ml.) by 1×10^6 r. It is of interest that although this highly contaminated honey was not completely sterilized by 1 and 2 million equivalent roentgens, the honey so treated did *not* cause AFB when fed to healthy colonies of bees, whereas the unirradiated infected honey produced extensive disease within one month.

Osmophilic Yeasts in Honey

Table I shows that the yeasts in the comb sections were completely killed by 0.5×10^6 r. Fermented honey was exposed to varying doses up to 2×10^6 r. and counts made for osmophilic yeasts (16). The results are given in Table IV and Fig. 2 and shown plotted as Curve 3 in Fig. 1. An original

TABLE IV

KILLING EFFECT OF HVE ON OSMOPHILIC YEAST IN HONEY

Dose $\times 10^6$ r.	Number	% Survivors
0	2040	100.0
0.025	800	40.0
0.05	440	22.0
0.075	210	10.5
0.1	55	2.7
0.25	1.5	0.007
0.5	0	0
1.0	0	0

contamination of 2040 yeasts per ml. was completely sterilized by 0.5×10^6 r. This exposure did not affect the organoleptic properties of the honey.

Honey inoculated with six different cultures of osmophilic yeasts with somewhat higher over-all concentration was treated with varying doses of HVE. The data from this experiment are shown in Table V and Curve 4 of

TABLE V

KILLING EFFECT OF HVE ON A MIXED CULTURE OF OSMOPHILIC YEAST IN HONEY

Dose $\times 10^6$ r.	Number of survivors	% Survivors
0	128,000	100.0
0.025	68,000	53.0
0.05	21,000	16.4
0.075	12,000	9.3
0.1	9500	7.4
0.25	2800	2.2
0.5	570	0.44
1.0	20	0.015
2.0	0	0

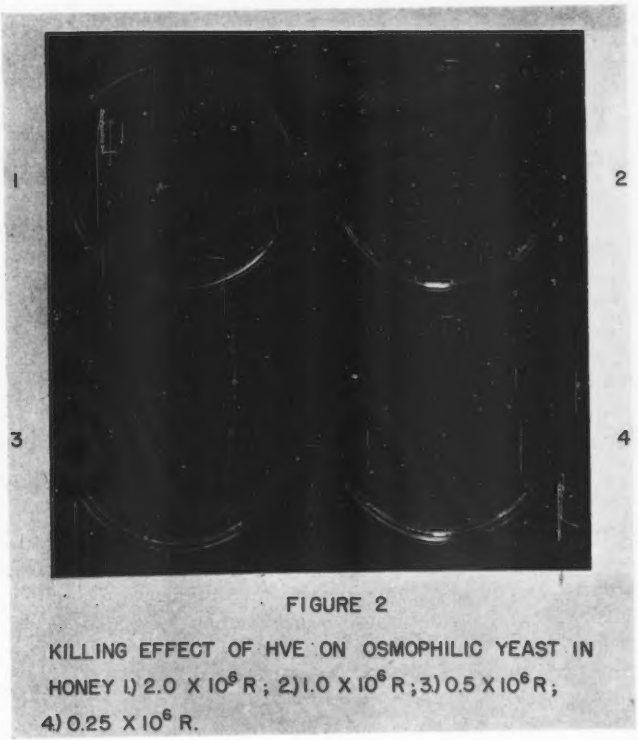

FIGURE 2

KILLING EFFECT OF HVE ON OSMOPHILIC YEAST IN HONEY 1) 2.0 X 10^6 R; 2) 1.0 X 10^6 R; 3) 0.5 X 10^6 R; 4) 0.25 X 10^6 R.

FIG. 2.

Fig. 1. The sharp break in the curve (Fig. 1) can be interpreted to indicate that there are at least two yeast types of different sensitivity to ionizing radiation in this mixture; one is three to four times more resistant than the other. The more resistant type is about as radioresistant as AFB spores.

No noticeable change in flavor was noted in honey treated with doses up to 6.0 million roentgen equivalents.

DISCUSSION

It was previously pointed out that normal comb containing capped or partially filled cells is too thick for penetration by an 800 kev. Because penetration is directly proportional to the accelerating voltage, irradiation from both sides by 2000 to 3000 kev. would provide sufficient penetration for used comb provided that the comb did not have, on opposite sides of the midrib, cells filled with honey or pollen that were in line. Technically, it appears that the method eliminates the contamination with AFB without destroying the usefulness of the comb.

Honey can be irradiated by passing it through the electron beam in a thin layer. A dose of 1 to 2×10^6 r., which is in the practical range, not only killed the osmophilic yeasts but also reduced *B. larvae* to such low numbers that infection of hives was eliminated. Whether such a method for sterilizing infected comb and honey is of economic interest depends upon many factors not considered here. It is to be clearly pointed out that, because of irradiation equipment considerations, the economics would *certainly not* justify use of this sterilization method on a small scale. Any considerations involving use of the method would probably have to be in terms of centrally located units or plants.

The shape of the survivor curve, a straight line on a semilog plot, fits one of the requirements advanced by Lea and others (13) that the lethal effect of the irradiation is due to a single ionization in a sensitive volume within the cell. Therefore, the dose necessary to completely sterilize a sample varies directly with the logarithm of the original number of contaminating organisms. This cannot be applied, of course, if more than one type of sensitivity to ionizing radiation is present. Results of tests with the mixed culture of osmophilic yeasts illustrate this point.

ACKNOWLEDGMENTS

The authors are indebted to Dr. A. G. Lochhead, Chief, Division of Bacteriology and Dairy Research, Department of Agriculture, Ottawa, for encouraging this work and providing the cultures of osmophilic yeasts; and to the technical staff of the Bee Division, Experimental Farms Service, for manipulating the colonies of bees. We are also indebted to Mr. J. S. Balwit of General Electric for his valuable help with the irradiation.

REFERENCES

1. CHARLTON, E. E. and WESTENDORP, W. F. Gen. Elec. Rev. 44: 652-661. 1941.
2. DUNN, C. G., CAMPBELL, W. L., FRAM, H., and HUTCHINS, A. J. Applied Phys. 19: 605-616. 1948.
3. FOSTER, J. W. and WYNNE, E. S. J. Bact. 55: 623-625. 1948.
4. FOSTER, J. W. and WYNNE, E. S. J. Bact. 55: 495-501. 1948.
5. HOLST, E. C. Am. Bee J. 86: 14. 1946.
6. HOLST, E. C. and STURTEVANT, A. P. J. Bact. 40: 723-731. 1940.
7. HUBER, W. D. Naturwissenschaften, 38: 21-29. 1951.
8. JOHNSON, J. P. J. Econ. Entomol. 40: 338-343. 1947.
9. JOHNSON, J. P. J. Econ. Entomol. 41: 314-318. 1948.
10. KATZNELSON, H. and LOCHHEAD, A. G. Sci. Agr. 27: 67-71. 1947.
11. LAWTON, E. J., BELLAMY, W. D., HUNGATE, R. E., BRYANT, M. P., and HALL, E. Science, 113: 380-382. 1951.
12. LAWTON, E. J., BELLAMY, W. D., HUNGATE, R. E., BRYANT, M. P., and HALL, E. Tech. Assoc. Pulp Paper Ind. In press.
13. LEA, D. E. Actions of radiations on living cells. The Macmillan Company, New York. 1947.
14. LOCHHEAD, A. G. Bee World, 14: 114-115. 1933.
15. LOCHHEAD, A. G. Can. J. Research, C, 15: 79-86. 1937.
16. LOCHHEAD, A. G. and HERON, D. A. Microbiological studies of honey. Dom. Can. Dept. Agr. Bull. No. 116. 1929.